Reference: *Biol Bull* **188**: 146–156. (April, 1995)

Microtubule-Based Movements During Ooplasmic Segregation in the Medaka Fish Egg (*Oryzias latipes*)

TAMIKA A. WEBB, WENDY J. KOWALSKI, AND RICHARD A. FLUCK*

Franklin and Marshall College, Department of Biology, Lancaster, Pennsylvania 17604

Abstract. We used time-lapse video microscopy to monitor the effects of cytochalasin D (CCD) and demecolcine on cytoplasmic streaming toward the animal pole of the medaka egg, the formation of the blastodisc at the animal pole, the movement of oil droplets in the cytoplasm toward the vegetal pole, and the saltatory movement of small cytoplasmic parcels toward the animal pole and vegetal pole. Cytochalasin D inhibited both cytoplasmic streaming toward the animal pole and the formation of the blastodisc, suggesting a role for microfilaments in these processes. However, CCD had no apparent effect on saltatory movement or on the movement of oil droplets toward the vegetal pole. Thus, the segregation of oil droplets toward the vegetal pole is not the result of the bulk movement of ooplasm toward the animal pole.

In eggs treated with demecolcine, oil droplets did not move toward the vegetal pole but instead floated to the uppermost portion of the egg, and saltatory movement was absent, suggesting that microtubules are required for these movements. The effects of demecolcine on oil droplet movement and saltatory movement could be reversed by irradiating the eggs with UV light (360 nm). Using indirect immunofluorescence, we showed that irradiation of demecolcine-treated eggs with UV light regenerated microtubules within the irradiated region.

The specificity of the mechanism responsible for the vegetal poleward movement of oil droplets was assessed by microinjecting droplets of five other fluids—mineral oil, silicone oil, vegetable oil, and two fluorinated aliphatic compounds—into the ooplasm. None of these fluids segregated with the endogenous oil droplets. These results suggest that a specific mechanism, probably involving microtubules, is responsible for the segregation of oil droplets to the vegetal pole.

Received 19 July 1994; accepted 11 January 1995.

* To whom correspondence should be addressed.

Introduction

The optical clarity, size (diameter = 1.2 mm), and year-round availability of the medaka fish egg make it a favorable system in which to study ooplasmic segregation. Ooplasmic segregation in this egg consists of the bulk flow of ooplasm toward the animal pole of the egg, the movement of oil droplets toward the vegetal pole, and the saltatory movement of small ooplasmic inclusions toward the animal and vegetal poles of the egg (Iwamatsu, 1973; Abraham *et al.*, 1993a), all of which take place more or less simultaneously. Formation of the blastodisc at the animal pole of zebrafish (*Danio rerio*) and loach (*Misgurnus fossilis*) eggs is inhibited by cytochalasin—cytochalasin B (CCB) in zebrafish (Katow, 1983) and cytochalasin D (CCD) in loach (Ivanenkov *et al.*, 1987). This inhibition suggests that microfilaments are involved in the movement of ooplasm toward the animal pole of these eggs. However, there are no reports of the effects of CCD on streaming or on formation of the blastodisc in the medaka egg. Thus, one objective of the present study was to monitor the effects of CCD on these phenomena in the medaka egg.

The possible involvement of microtubules in segregation in the medaka egg has been explored more fully (Abraham *et al.*, 1993a, 1993b). A network of microtubules is organized during the period of ooplasmic segregation in the medaka (Abraham *et al.*, 1993b), and this network is absent from eggs incubated with a microtubule poison (Abraham *et al.*, 1993b). Moreover, three microtubule poisons—colchicine, demecolcine, and nocodazole—inhibit the normal movement of oil droplets toward the vegetal pole, eliminate the saltatory motion of small inclusions, and slow the growth of the blastodisc; eggs treated with β-lumicolchicine, an inactive derivative of colchicine, segregated normally (Abraham *et al.*, 1993a). The second objective of the present study was to determine

whether the inhibitory effects of one of these poisons (demecolcine) on microtubules and ooplasmic segregation could be reversed by illuminating the eggs with ultraviolet light (360 nm), which photolyzes demecolcine, converting it to lumidemecolcine, a molecule that is not a microtubule poison (Aronson and Inoué, 1970). This "Colcemid-UV method" (Colcemid is a trade name for demecolcine) has been used by a number of investigators to study microtubule-mediated movements (Sluder, 1976; Hiramoto *et al.*, 1984; Hamaguchi and Hiramoto, 1986; Ladrach and La Fountain, 1986).

The suggestion that microtubules are involved in the segregation of oil droplets toward the vegetal pole is inconsistent with the hypothesis that this segregation is caused simply by the bulk flow of ooplasm in the opposite direction, that is, toward the animal pole (Sakai, 1965). This hypothesis can be tested directly by determining whether oil droplets segregate toward the vegetal pole when there is no bulk flow of ooplasm toward the animal pole. Thus, another objective of the present study was to monitor the effects of CCD on the segregation of oil droplets toward the vegetal pole.

Sakai's hypothesis was based in part on the observation that when droplets of three oils—vegetable oil, liver oil, and mineral oil—were injected into the ooplasm of medaka eggs, they segregated toward the vegetal pole along with endogenous droplets (Sakai, 1965). However, it is possible that the injected droplets simply *floated* toward the vegetal pole, because the eggs in the experiments were all oriented with their vegetal pole uppermost. Thus, another objective of the present study was to monitor the movement of injected droplets of five fluids that differ widely in their chemical and physical properties; the eggs in the present study were oriented with either their animal pole or their vegetal pole uppermost.

A preliminary account of these findings has been published (Webb and Fluck, 1993).

Materials and Methods

Biological material

The dissection of gonads from breeding medaka, the preparation of gametes, and the *in vitro* fertilization of eggs have been previously described (Yamamoto, 1967; Abraham *et al.*, 1993a). To indicate the relative temporal position of these events, we used a normalized time (T_n) scale in which the time between fertilization and the beginning of cytokinesis is 1 unit.

Effects of cytochalasin D on segregation

Stock solutions of CCD (Sigma) in dimethylsulfoxide (DMSO, Fisher) were diluted 100-fold to make working solutions. Four eggs were placed in a small Syracuse watch glass containing 500 μl of either 1% DMSO in buffered saline solution (BSS: 111 mM NaCl; 5.37 mM KCl; 1.0 mM $CaCl_2$; 0.6 mM $MgSO_4$; 5 mM HEPES, pH 7.3) or 10 μg ml^{-1} CCD/1% DMSO in BSS and incubated for 1 h at room temperature (24–25°C). The eggs were fertilized by pipetting 50 μl of a fresh sperm suspension, prepared by mincing testis in BSS, onto the eggs and stirring quickly. Fertilized eggs were transferred to a microscope slide on which a cover glass was supported by four pillars of petroleum jelly (Abraham *et al.*, 1993a). The volume of the blastodisc was measured with an image analysis program (Microcomp Planar Morphometry, Southern Micro Instruments; Abraham *et al.*, 1993a). To monitor cytoplasmic streaming, we oriented the eggs with their animal pole–vegetal pole axis parallel to the surface of the slide, placed the slide on the stage of a compound microscope (Nikon Optiphot), and used time-lapse video microscopy via a 40× phase-contrast objective and a Dage/MTI Newvicon camera to record ooplasmic movements. On playback, the positions of five particles in each egg at $T_n \approx 0.35$ were mapped onto acetate sheets (Abraham *et al.*, 1993a). At the end of recording, the eggs were washed twice with embryo reading medium (17 mM NaCl; 0.4 mM KCl; 0.3 mM $CaCl_2$; 0.67 mM $MgSO_4$; 0.001 g/l methylene blue) and transferred to a petri dish containing 3 ml of embryo rearing medium. Subsequent development of the eggs was monitored for several days. A total of 111 eggs from six females were used in these experiments; we monitored ooplasmic segregation closely in 30 eggs.

Effects of demecolcine on segregation

Working solutions of demecolcine (N-deacetyl-N-methyl-colchicine; Sigma) were prepared by diluting (at least 100×) an aqueous stock solution of 0.35 mM demecolcine. Eggs were placed in BSS containing the appropriate concentration of demecolcine and incubated for 1 h at room temperature in dim light. Typically three eggs were incubated in 375 μl or four eggs in 500 μl of medium. To fertilize the eggs, we minced a portion of testis in 200 μl of BSS and pipetted 50 μl of sperm suspension onto the eggs in the appropriate (drug-containing) medium. Eggs were then transferred to a coverglass-slide assembly for microscopy. To monitor ooplasmic segregation in these eggs, we transilluminated the egg with light from a quartz-halogen lamp, using a heat filter (KG5) and a 486 DF32 filter (Omega Optical). Movements were recorded by time-lapse video microscopy with a Dage/MTI SIT camera.

In some experiments, we treated eggs with both CCD (10 μg ml^{-1}) and demecolcine (0.35 μM).

Irradiating eggs with ultraviolet light

To irradiate eggs with UV light, we used an Osram 100 W mercury arc lamp. The light from the lamp was

passed through a custom filter cube (Omega Optical) containing a 360 DF 40 filter and a DC 405 dichroic mirror. An octagonal diaphragm was used to control the size of the light beam. To reduce the light intensity in some experiments, we used either an ND16 filter (Nikon, which reduced the intensity ≈ 94%) or an ND 1.5 filter (Omega Optical, which reduced the intensity ≈ 97%). The light was projected onto the egg via one of three objective lenses (all from Nikon): Plan 4×, N.A. = 0.1; Fluor/Ph 2 DL 10×; N.A. = 0.5; Fluor/Ph 3 DL 20×, N.A. = 0.75. Specific regions of the egg—animal pole, vegetal pole, equatorial region—were illuminated either *en face* or *en profil.* In most of the experiments described herein, we used the 10× objective lens and irradiated an octagonal region having a diameter of 475 μm and an area of 1.9×10^5 μm^2. Light intensity was measured with a UVX radiometer with a long wave sensor (UVP, Inc.). Given a light intensity of 523 $\mu W\ cm^{-2}$ and assuming that all the UV light was of wavelength 360 nm, we calculated an incident light intensity of 4.9×10^9 quanta s^{-1} μm^{-2} (10× objective lens; no neutral density filter). To monitor the subsequent development of eggs, we washed them twice with embryo rearing medium and transferred them to a petri dish containing 3 ml of embryo rearing medium. A total of 292 eggs from 18 females were used in these experiments; we monitored ooplasmic segregation closely in 96 eggs.

Indirect immunofluorescence

The methods for fixing eggs and performing indirect immunofluorescence assays for alpha-tubulin have been described previously (Gard, 1991; Abraham *et al.*, 1993b). Briefly, the eggs were fixed for 4 h in a mixture of formaldehyde (Electron Microscopy Sciences) and glutaraldehyde (Electron Microscopy Sciences) at room temperature and then in cold (−20°C) methanol; treated with sodium borohydride; incubated with a monoclonal mouse anti-alpha-tubulin antibody (ICN, DM1A; diluted 1:250 with TBS (155 m*M* NaCl; 10 m*M* Tris-Cl, pH 7.4; 0.1% Nonidet P-40) containing 2% bovine serum albumin; and incubated with a secondary antibody (Organon Teknika, rhodamine-conjugated goat anti-mouse IgG, diluted 1:25 with TBS containing 2% bovine serum albumin). Control eggs were not incubated with the primary antibody. Eggs that were in a solution of demecolcine and being irradiated with UV light were fixed on the stage of the microscope for 5 min before being transferred to a vial of fixative. To stain nuclei, we incubated the eggs for 30 min in a solution of Hoechst 33258 (Sigma, 10 $\mu g\ ml^{-1}$) in TBS. A total of 68 eggs from 11 females were used in these experiments.

Microinjecting fluids into medaka eggs

Using methods described previously (Fluck *et al.*, 1991, 1992, 1994), droplets of fluid were microinjected into the ooplasm between 35° and 90° arc from the animal pole. Of the 25 eggs used in these experiments, 21 were parthenogenetically activated by the injection needle, and four others were fertilized after injection. Similar results were obtained with parthenogenetically activated and fertilized eggs. Droplets of five fluids were injected: mineral oil, density = 0.84 $g\ ml^{-1}$; vegetable oil, density = 0.9 $g\ ml^{-1}$; silicone fluid (Sigma), density = 1.05 $g\ ml^{-1}$; and two fluorinated aliphatic compounds, FC-77 and FC-3275 (3M), density = 1.78 $g\ ml^{-1}$. Although droplets of silicone fluid and the fluorinated compounds could be distinguished visually from endogenous oil droplets, we stained the vegetable oil and mineral oil with either Sudan black B or Sudan III to identify them.

To monitor the movement of the injected droplets, we used one of two techniques. In the first, we placed an egg on a small mound of Dow Corning high vacuum grease in the bottom of a rectangular plastic spectrophotometer cuvette and used two video cameras and two time-lapse videocassette recorders to record the movement of the droplets simultaneously from two perspectives: a polar view and a side view. In the second technique, we glued the egg to the bottom of a petri dish (diam., 35 mm) with Cel-Tak (Bio-Polymers, Inc.) and placed a right-angle prism (Melles-Griot) next to the egg. The petri dish was then placed on the stage of an inverted microscope (Nikon Diaphot) and viewed either directly (polar view) or indirectly via the prism (side view). To view the egg indirectly, we illuminated it from the side, using a fiber optic cable.

Results

Effects of cytochalasin D and demecolcine on ooplasmic segregation and development

Eggs treated with 1% DMSO formed a blastodisc (volume at $T_n \approx 0.85$–1.0 = 15.4 ± 2.0 nl, $\bar{X} \pm$ SD, $N = 7$ eggs; Fig. 1A), displayed cytoplasmic streaming (speed of parcels toward the animal pole = 9.00 ± 5.52 $\mu m\ min^{-1}$, $\bar{X} \pm$ SEM, $N = 7$ eggs), and developed normally. In contrast, eggs treated with CCD (10 $\mu g\ ml^{-1}$) had essentially no cytoplasmic streaming (velocity = −0.66 ± 0.84 $\mu m\ min^{-1}$, $\bar{X} \pm$ SEM, $N = 5$ eggs), did not form a blastodisc (Fig. 1B), and did not undergo cell division. CCD had no apparent effect on the segregation of oil droplets toward the vegetal pole (Fig. 1B) or on the saltatory movement of particles toward either the animal or vegetal pole.

The effects of demecolcine on the eggs were those previously described by Abraham *et al.* (1993a): (1) oil droplets did not segregate to the vegetal pole but instead floated to the portion of the egg that was uppermost during the experiment—equator (Fig. 1C), animal pole (Fig. 2C and D), or vegetal pole (not shown); (2) a smaller-than-normal

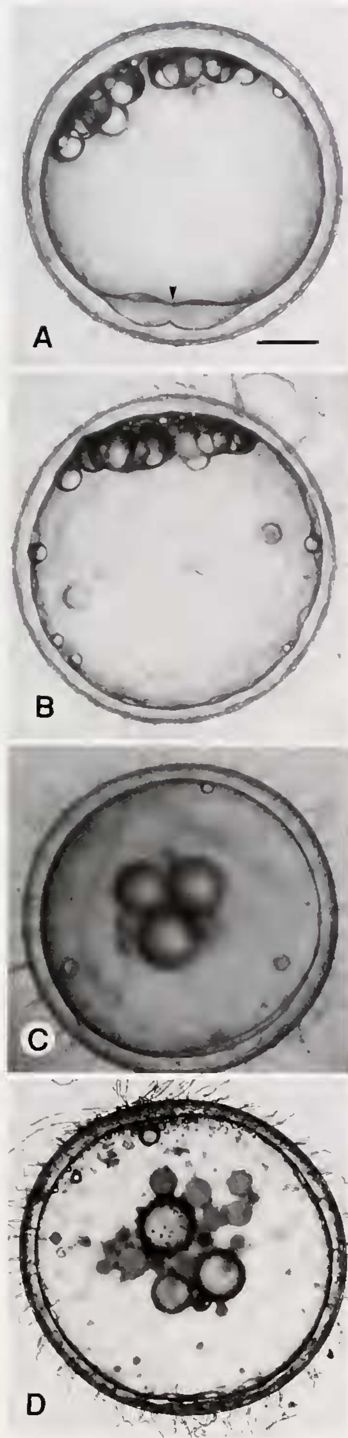

Figure 1. The effects of CCD and demecolcine on formation of the blastodisc and movement of oil droplets. The animal pole is near the bottom and the vegetal pole near the top of each figure. (A) T_n = 1.24. DMSO (1% in BSS) had no apparent effect on the formation of the blastodisc at the animal pole of the egg, the movement of oil droplets toward the vegetal pole, or cell division (note the cleavage furrow, arrowhead). (B) T_n = 1.34. CCD (10 μg ml^{-1}) inhibited formation of the blastodisc but had no apparent effect on the segregation of oil droplets toward the vegetal pole. (C) T_n = 1.0. This egg was treated with 1.0 μM demecolcine. Note the small blastodisc at the animal pole (compare with Figure 1A) and that most oil droplets did not move toward the vegetal pole but floated to the top of the egg. (D) T_n = 0.73. This egg was treated with both CCD and demecolcine (0.35 μM). Note the absence of a blastodisc at the animal pole and that the oil droplets have floated to the top of the egg. Scale bar, 250 μm.

blastodisc formed (Fig. 1C); (3) saltatory motion was absent; and (4) the eggs did not cleave.

In eggs treated with both CCD and demecolcine, we saw no saltatory movement, no cytoplasmic streaming, and no movement of oil droplets toward the vegetal pole (Fig. 1D). These eggs also did not cleave (Table I).

Reversal of the inhibitory effects of demecolcine on ooplasmic segregation and embryonic development

When demecolcine-treated eggs were irradiated with UV light, the inhibitory effects of demecolcine were reversed: oil droplet movement and saltatory movement were restored; and the eggs subsequently cleaved, formed an embryonic axis, and hatched. The effects of UV irradiation were less pronounced at higher concentrations of demecolcine. For example, of 11 eggs treated with 0.35 μM demecolcine and irradiated *en face* at the animal pole (2.8×10^8 quanta s^{-1} μm^{-2}), vegetal pole, or equator, all 11 cleaved and developed an embryonic axis; while of three eggs treated with 3.5 μM demecolcine and irradiated under similar conditions, all three cleaved but none developed an embryonic axis. The effects of UV irradiation were also less pronounced when we irradiated the eggs *en profil* instead of *en face*. For example, of five eggs treated with 0.35 μM or 1.0 μM demecolcine and irradiated *en face* near the equator (2.8×10^8 quanta s^{-1} μm^{-2}), all five cleaved and developed an embryonic axis. However, of three eggs treated similarly but irradiated *en profil* at the equator, none cleaved.

Apparently normal oil droplet movement was restored when we irradiated the animal pole or equatorial region *en face*, with oil droplets near the animal pole or equator moving away from the animal pole and toward the vegetal pole more or less along meridian lines (Fig. 2A–F). Under appropriate experimental conditions (1.0 μM demecolcine; irradiation with low intensities of UV light), this reversal of the effects of demecolcine on oil droplet movement was restricted to the portion of the egg that was irradiated. In such eggs, oil droplets outside the irradiated region floated to the top of the egg and accumulated at the edge of the irradiated region, while droplets within the irradiated region moved out of the irradiated region and toward the vegetal pole (Fig. 2G and H).

When we irradiated an equatorial region *en profil*, oil droplets in the irradiated region moved toward the vegetal pole, while oil droplets in the rest of the egg either floated

to the top of the egg or did not move at all (Fig. 2I and J). In eggs irradiated *en profil* at the equator, we also saw a small accumulation of ooplasm at the animal-pole side of the irradiated region (Fig. 2I and J).

The effects of UV irradiation on oil droplet movement and other phenomena clearly extended beyond the irradiated region when we used a lower concentration of demecolcine or a higher intensity of UV light. For example, eggs irradiated *en face* with a high intensity of UV light near their equator often underwent cleavage and formed an embryonic axis. In addition, oil droplets in such eggs moved beyond the edge of the irradiated region (Fig. 2K).

The movements of droplets within the irradiated regions were very similar to those in control (not treated with demecolcine) eggs; that, is roughly along meridian lines and away from the animal pole (Fig. 3).

Segregation appeared normal in medaka eggs that were irradiated with UV light intensities ≤ about 530 μW cm^{-2} (4.9×10^9 quanta s^{-1} μm^{-2}) but not treated with demecolcine (not shown). However, in preliminary experiments, we found that higher light intensities (≥1100 μW cm^{-2}, 1.0×10^{10} quanta s^{-1} μm^{-2}) inhibited both the growth of the blastodisc and the movements of oil droplets.

Microtubule regeneration by ultraviolet irradiation

We saw no microtubules in eggs that were treated with demecolcine but not irradiated with UV light before fixation (Fig. 4A). Moreover, we saw no or very few microtubules outside the irradiated region of eggs that were irradiated with UV light (Fig. 4B and C). However, the UV-irradiated regions contained microtubules that in number and orientation were very similar to those in control eggs that had not been treated with demecolcine (Fig. 5).

The movement of droplets of injected fluids

Droplets of mineral oil were injected into five eggs, which were oriented with their animal pole uppermost. In two of them, the injected droplets fused with native droplets, and the hybrid droplets moved toward the vegetal pole. In two eggs in which the injected droplets were not observed to fuse with native droplets, the injected droplets moved up, toward the animal pole (Fig. 6A); and in another egg, the droplet of mineral oil did not move at all, even though nearby native droplets did move toward the vegetal pole. The eggs into which we injected FC-77 (four eggs), FC-3275 (one egg), and silicone oil (four eggs), were oriented with their vegetal pole uppermost. In all these eggs, the droplets of injected fluids moved toward the animal pole and came to rest in the blastodisc (Fig. 6B–D).

We injected vegetable oil into 11 eggs and oriented them with their animal pole uppermost. In eight of these eggs, the injected droplet fused with endogenous oil droplets, and these hybrid droplets segregated toward the vegetal pole. However, in one egg in which the injected droplet was not observed to fuse with endogenous droplets, the injected droplet moved up, toward the animal pole, and came to rest near the blastodisc, while nearby endogenous droplets moved by it in the opposite direction on their way to the vegetal pole. In two cases in which we injected vegetable oil, the injected droplets appeared to follow larger endogenous droplets that they were touching.

In the case of every fluid—vegetable oil, mineral oil, FC77, FC3275, and silicone oil—the movements of nearby or immediately adjacent endogenous oil droplets appeared to be unaffected by the presence of the injected fluid. In other words, endogenous droplets, moving toward the vegetal pole, passed by the droplets of injected fluid, which were either stationary or moving toward the animal pole.

Discussion

The inhibition of cytoplasmic streaming and formation of the blastodisc by CCD in medaka eggs is consistent with data obtained from other teleost eggs (Katow, 1983; Ivanenkov *et al.*, 1987) and from eggs of other organisms, including ascidians (Sawada and Osanai, 1981; Jeffery,

Table I

Cytoplasmic streaming, saltatory movement, oil droplet movement, and cleavage in the presence of CCD (10 μg ml^{-1}), demecolcine (0.35 μM), and CCD/demecolcine

Treatment	Cytoplasmic streaming	Oil droplet movement toward vegetal pole	Saltatory movement	Cleavage
Cytochalasin D	No	Yes	Yes[a]	No
Demecolcine	Yes	No[b]	No	No
Cytochalasin D and demecolcine	No	No	No	No

[a] Saltatory movement toward both the animal and vegetal poles of the egg.
[b] Oil droplets floated to the top of the egg.

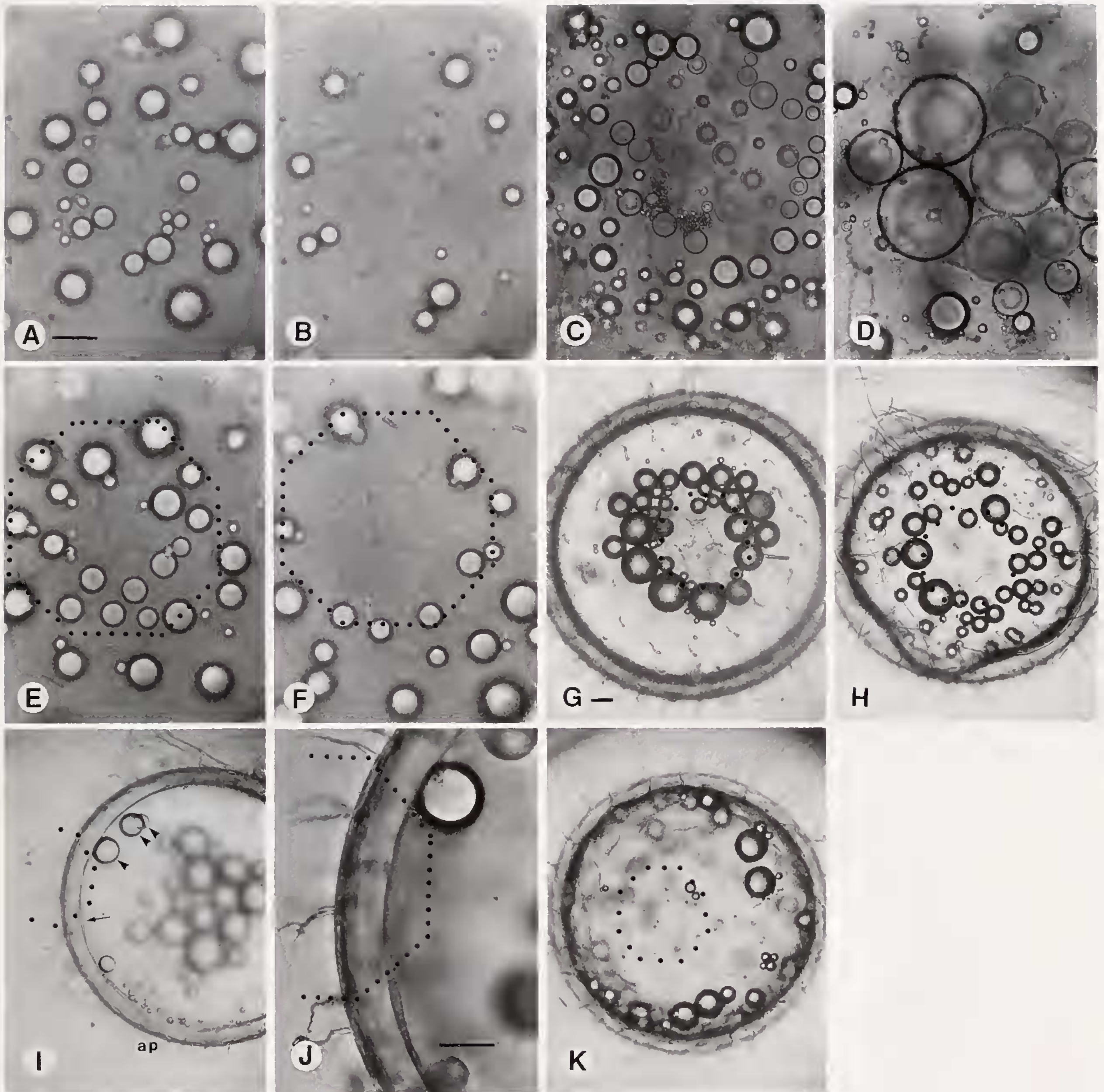

Figure 2. Ultraviolet light reverses the effect of demecolcine on oil droplet movement. (A) and (B) show oil droplets near the animal pole of a control egg (not treated with demecolcine) at $T_n = 0.3$ and 0.5, respectively. Note that many oil droplets have left this region of the egg by $T_n = 0.5$. In (C), where $T_n = 0.14$, and (D), where $T_n = 0.65$, oil droplets have floated to the top and accumulated at the animal pole of an egg treated with 0.35 μM demecolcine and oriented with animal pole uppermost. An egg that was treated with 1.0 μM demecolcine and irradiated with UV light (7.6×10^8 quanta s^{-1} μm^{-2}) *en face* at the animal pole is shown at $T_n = 0.3$ in (E) and $T_n = 0.5$ in (F). The edges of the diaphragm are shown (●●●). Note that the oil droplets have moved away from the animal pole. The egg shown in (G) at $T_n = 0.75$ was incubated in 1.0 μM demecolcine and irradiated *en face* at its animal pole (2.8×10^8 quanta s^{-1} μm^{-2}). The ring of oil droplets is the result of (1) oil droplets outside the irradiated region floating to the top of the egg (toward the viewer) and accumulating at the edge of the irradiated region, and (2) oil droplets within the irradiated region moving to the edge of the irradiated region. This embryo developed normally and hatched. The egg shown in (H) at $T_n = 0.67$, was incubated in 1.0 μM demecolcine and illuminated *en face* at its equator (2.8×10^8 quanta s^{-1} μm^{-2}). As in Figure 2G, note the absence of oil droplets from the irradiated region and the presence of a ring of oil droplets at the edge of the irradiated region. This egg cleaved and formed an embryonic axis, but morphogenesis was very abnormal and the embryo did not hatch. (I) at $T_n = 1.1$, shows an egg that was treated with 1.0 μM demecolcine and irradiated *en profil* near its equator (2.8×10^8 quanta s^{-1} μm^{-2}). Note the accumulation of most oil droplets at the top of the egg (toward the viewer and out of focus) and the formation of a smaller than normal blastodisc at the animal pole. Three oil droplets

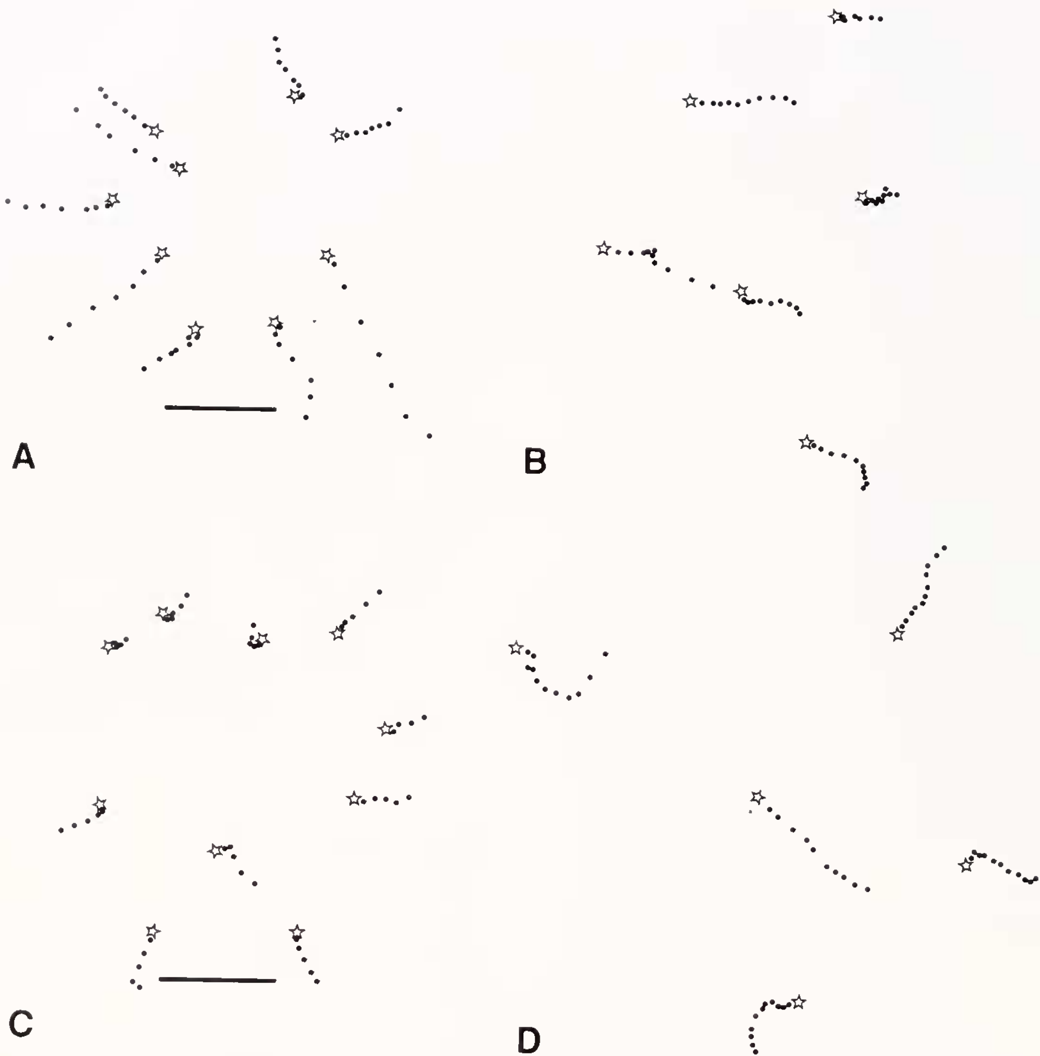

Figure 3. Oil droplets within UV-irradiated regions move toward the vegetal pole. A star marks the starting position of each droplet, and black circles mark the positions at 2-min intervals. The eggs in (A) and (B) were untreated controls shown from either the animal pole (A) or the equator (B); the eggs shown in (C) and (D) were treated with 1.0 μM demecoleine and irradiated *en face* (3.3×10^8 quanta s^{-1} μm^{-2}) at either the animal pole (C) or equator (D). Oil droplets near the animal pole (near the center of the figure) in (A) and (C) moved away from that pole more or less along meridian lines; droplets near the equator in (B) and (D) moved toward the vegetal pole (to the right). Scale bars: (A) and (B), 200 μm; (C) and (D), 100 μm. (A) and (B) were printed at one magnification and (C) and (D) at another.

1984) and an oligochaete (Shimizu, 1982). The inhibition suggests that the formation of the blastodisc in the medaka egg is the result of the streaming of ooplasm to the animal pole and that microfilaments are involved in this streaming. This suggestion should be pursued by staining eggs with fluorescently labeled phalloidin during segregation.

The solation-contraction coupling hypothesis (Janson and Taylor, 1993) provides a model for how this stream-

---

Figure 2. (*Continued*) (arrowheads) were observed to move through the irradiated region toward the vegetal pole. The small accumulation of ooplasm at the animal pole side of the irradiated region (arrow) is shown at higher magnification in (J). The egg in (K), where $T_n = 0.60$, was treated with 1.0 μM demecoleine and irradiated at the animal pole *en face* with a high intensity of UV light (5.0×10^9 quanta s^{-1} μm^{-2}). Note that oil droplets have moved beyond the edges of the irradiated region. Scale bar, 100 μm. (A–F) are printed at the same magnification, (G–I) at another, and (J) at another.

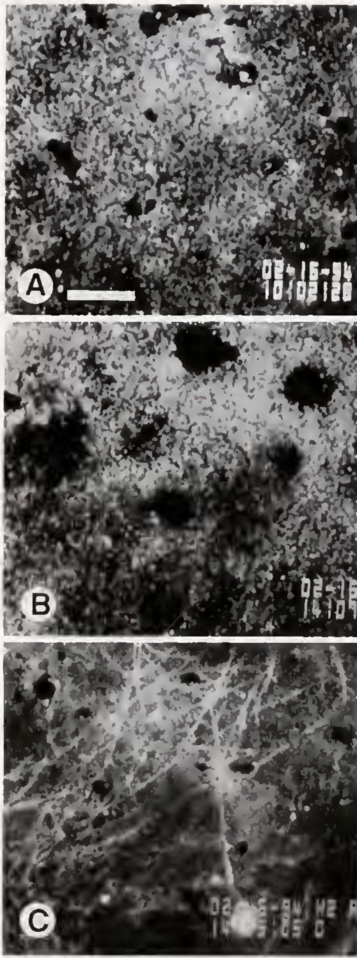

Figure 4. Microtubules in eggs treated with demecolcine. All three photographs were taken near the equator of the egg. The egg shown in (A) was treated with 3.5 μM demecolcine but was not irradiated with UV light. Note the absence of microtubules. Of 17 such eggs we observed, we found no microtubules in 15 and a few scattered ones in 2. (B) and (C) show ooplasm near the equator in eggs that were treated with 3.5 μM demecolcine and irradiated with UV light (5.2×10^8 quanta s^{-1} μm^{-2}) *en face* at the animal pole (B) or *en profil* at the vegetal pole (C). Note the apparent absence of microtubules in (B), a typical field, and the relatively few even in (C), which had the most microtubules that we observed outside an irradiated region (compare with Fig. 5). Scale bar, 10 μm.

ing, which is likely driven by the interaction of actin and myosin, could be organized and driven by the zone of elevated cytosolic Ca^{2+} present at the animal pole throughout the period of segregation (Fluck *et al.*, 1992). Dissipation of this zone of elevated Ca^{2+} inhibits formation of the blastodisc (Fluck *et al.*, 1992, 1994).

The experiments with CCD directly test the hypothesis that the movement of oil droplets toward the vegetal pole is caused by the movement of ooplasm in the opposite direction—toward the animal pole. The results are not consistent with this hypothesis, because oil droplets moved toward the vegetal pole even when we did not observe any cytoplasm streaming to the animal pole. Other data from the present study also conflict with the hypothesis. Specifically, in demecolcine-treated eggs oriented with their animal pole uppermost, oil droplets collected at the animal pole even as ooplasm was also streaming in that direction.

Moreover, the failure of injected droplets of several other fluids—fluorinated aliphatic compounds, silicone fluid, mineral oil, and vegetable oil—consistently to move away from the animal pole and toward the vegetal pole during segregation is inconsistent with the hypothesis. This failure was probably not due to a toxic effect of the fluids on nearby cell components, because the movements of adjacent endogenous droplets appeared to be unaffected by proximity to a droplet of injected fluid. The behavior of the injected droplets also suggests a specificity of the mechanism responsible for the movement of endogenous oil droplets.

We have previously suggested that microtubules are required for the segregation of oil droplets to the vegetal pole of the medaka egg (Abraham *et al.*, 1993a). In the present study, we explored the connection between microtubules and oil droplet movement further by using the "Colcemid-UV" method to photolyze demecolcine in specific regions of the egg and thus presumably create conditions that might support microtubule polymerization (Dustin, 1984, ch. 5; Bray, 1992, p. 207). We found that irradiation of the egg with UV light regenerated both microtubules and apparently normal oil droplet movement within the irradiated region of the egg. These results are consistent with a role for microtubules in the movement of oil droplets. To determine whether the microtubule-based motor protein, kinesin, has any role in oil droplet movement, we are planning experiments in which we will microinject anti-kinesin antibodies (Ingold *et al.*, 1988) into medaka eggs.

Microtubule poisons also disrupt intermediate filaments in a number of cells (Knapp *et al.*, 1983; Hunt and Davis, 1990; Pasdar *et al.*, 1992), so it is possible that some of the effects we observed were caused by such disruption in the medaka egg. We are investigating whether intermediate filaments are present in the medaka egg and can be disrupted by microtubule poisons.

Although the effects of UV irradiation on oil droplet movement could be localized under appropriate experimental conditions, the effects of irradiation sometimes clearly extended beyond the irradiated region. For example, eggs that were irradiated *en face* at their equator usually cleaved and formed an embryonic axis. These results suggest that UV irradiation at the equator lowered the concentration of demecolcine in the animal pole region (where cell division occurs) enough to permit the polymerization of tubulin. Moreover, oil droplets often

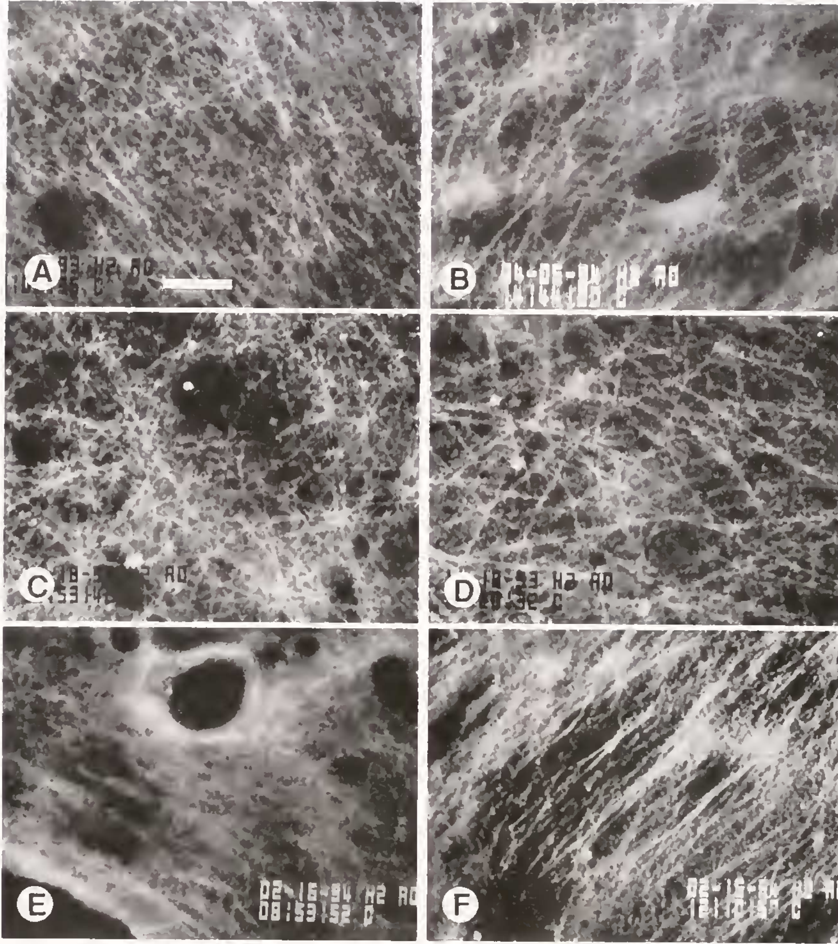

Figure 5. UV light reverses the effect of demecolcine on tubulin polymerization. The eggs shown in (A), (C), and (E) were neither treated with demecolcine nor irradiated with UV light; those shown in (B), (D), and (F) were treated with 3.5 μM demecolcine and irradiated with UV light (5.2×10^8 quanta s^{-1} μm^{-2}) either *en face* at the animal pole (B) or equator (D) or *en profil* at the vegetal pole (F). All eggs were fixed at $T_n = 0.3$. Near the animal pole, in (A) and (B), and the equator, in (C) and (D), a dense network of microtubules having no apparent preferred orientation is present, but near the vegetal pole, in (E) and (F), most of the microtubules are parallel to each other. *En profil* and *en face* irradiation gave identical results in all three regions of the egg. Scale bar, 10 μm.

moved beyond the edge of the UV-irradiated region, suggesting that microtubules that formed in the irradiated region subsequently extended beyond this region. Irradiation of the egg *en face* likely photolyzed demecolcine in the ooplasm on both the near side of the egg and its antipode as well as in the portion of the yolk vacuole that is in the light path. As molecules of demecolcine were inactivated within the irradiated region and the product diffused away from it, molecules of demecolcine in adjacent, unirradiated regions diffused into the irradiated region. In other words, the irradiated region acted as a demecolcine sink, and eventually the concentration of demecolcine decreased throughout the egg. The spatiotemporal pattern of the change in demecolcine concentration in an irradiated egg presumably depends on at least three variables: (a) the initial concentration of demecolcine in the egg, (b) the intensity of the UV light, and (c) the volume of the irradiated region. Our results demonstrate that all three variables affected the extent to which UV irradiation reversed the effects of demecolcine on oil droplet movement, cleavage, and subsequent development.

Irradiation of control medaka eggs with UV light (360 nm) at intensities adequate to regenerate microtubules and normal movement of oil droplets in poisoned eggs had no apparent effect on ooplasmic segregation. These results are consistent with those of other studies in which UV light has been used to modify amphibian development (Grant and Wacaster, 1972; Scharf and Gerhart, 1980; Holwill *et al.*, 1987). In all these studies, the

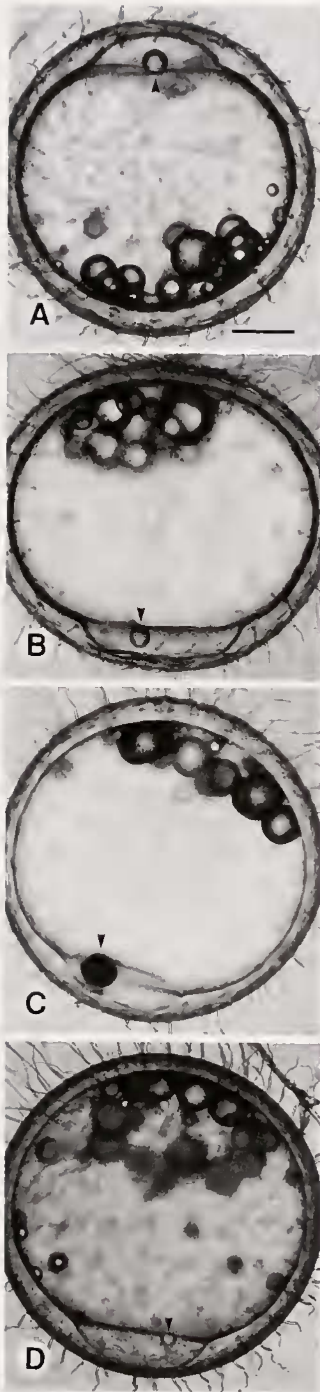

Figure 6. Behavior of injected droplets of fluids. Droplets of fluids were injected into unfertilized eggs, which were parthenogenetically activated by the injection. The egg in (A) was oriented with its animal pole uppermost; all others had vegetal pole uppermost. In all four eggs, the droplets of injected fluid (arrowheads) moved toward the animal pole of the egg and came to rest in the blastodisc at the boundary between the blastodisc and the yolk vacuole. (A) Mineral oil stained with Sudan black B, (B) FC-77, (C) FC-3275, (D) silicone fluid. Scale bar, 250 μm.

predominant wavelength emitted by the lamps was about 254 nm. Moreover, an action spectrum of the effects of UV irradiation on the inactivation of components required for neural induction in the amphibian egg showed a peak at 280 nm and a decline toward both 250 and 320 nm (Youn and Malacinski, 1980). However, irradiation of cells with high-intensity near-UV light (360 nm, 2.5 mW cm^{-2}, about 5-fold higher than that used in the present study) disrupts f-actin in epithelial cells (Rafferty *et al.*, 1993). Although we saw an inhibition of segregation at such a high UV light intensity in preliminary experiments, UV light intensities sufficient to regenerate microtubules and normal oil droplet movement had no apparent effect on ooplasmic segregation in the medaka egg.

That we could regenerate microtubules and oil droplet movement near the animal pole by irradiating this region *en face* is not surprising, given the apparent presence of a microtubule-organizing center there (Abraham *et al.*, 1993b). More interesting is the observation that we could regenerate microtubules and oil droplet movement near the equator region and could regenerate microtubules at the vegetal pole. This result is consistent with Sakai's observation (1964) that oil droplets will segregate toward the vegetal pole in vegetal halves of eggs that have been surgically separated from their animal halves. This result suggests the presence of microtubule-organizing centers outside the animal pole region of the medaka egg. In *Xenopus laevis* eggs, the biochemical basis for such an ability may be the presence of γ-tubulin in cortical cytoplasm in the vegetal hemisphere of the egg (Gard, 1994). We are currently pursuing this question.

Finally, we note that when we irradiated an equatorial region of an egg *en profil*, ooplasm accumulated at the animal pole side of the irradiated region, suggesting that microtubules are involved not only in the movement of oil droplets toward the vegetal pole but also in the movement of components of the ooplasm toward the animal pole. Though the growth of the medaka blastodisc is slowed by microtubule poisons (Abraham *et al.*, 1993a), no specific effect of these poisons on the movement of the ooplasm or its constituents toward the animal pole has been reported. However, here we have described the saltatory movement of subcellular parcels toward the animal pole and its inhibition by demecolcine. This movement was especially apparent in eggs treated with CCD, which displayed no cytoplasmic streaming; this streaming obscures the component of saltatory movement directed toward the animal pole. Ooplasm in and near these irradiated interpolar regions appeared to segregate along the same axis as that of the entire egg: oil droplets moved toward the vegetal pole and certain other components of the ooplasm toward the animal pole. These results suggest that a local mechanism, operating with a predetermined polarity, is responsible for the segregation of ooplasm in

the medaka egg. This suggestion is consistent with results obtained by Gilkey (1983), who achieved local activation of medaka eggs by injecting them with Ca/EGTA mixtures. He reported that oil droplets in such eggs moved to the vegetal-pole side of the activated region and a small amount of ooplasm accumulated at the animal pole side. We are seeking to determine whether these localized regions of segregation in the medaka egg are accompanied by polar zones of elevated cytosolic [Ca^{2+}] like those present at the poles of the egg during segregation (Fluck *et al.*, 1992).

Acknowledgments

Supported by NSF DCB-9017210 and NSF MCB-9316125 to RAF, Franklin and Marshall College's Hackman Scholar Program, and a grant from GTE to Franklin and Marshall College. We thank Shinya Inoué for suggesting the use of the Colcemid-UV method and for his advice in the selection of fluids for microinjection into the eggs, Vivek Abraham for helpful discussions, Richard Elinson for telling us about the paper by Youn and Malacinski, and Valerie Stone for improving the text of the manuscript.

Literature Cited

Abraham, V. C., S. Gupta, and R. A. Fluck. 1993a. Ooplasmic segregation in the medaka *(Oryzias latipes)* egg. *Biol Bull* **184:** 115–124.

Abraham, V. C., A. L. Miller, L. F. Jaffe, and R. A. Fluck. 1993b. Cytoplasmic microtubule arrays in *Oryzias latipes* (medaka) eggs during ooplasmic segregation. *Biol. Bull.* **185:** 305–306.

Aronson, J., and S. Inoué. 1970. Reversal by light of the action of *N*-methyl, *N*-desacetyl colchicine on mitosis. *J. Cell Biol.* **45:** 470–477.

Bray, D. 1992. *Cell Movements.* Garland Publishing, Inc., New York.

Dustin, P. 1984. *Microtubules*, 2nd ed. Springer-Verlag, Berlin.

Fluck, R. A., A. L. Miller, and L. F. Jaffe. 1991. Slow calcium waves accompany cytokinesis in medaka fish eggs. *J. Cell Biol.* **115:** 1259–1265.

Fluck, R. A., A. L. Miller, and L. F. Jaffe. 1992. High calcium zones at the poles of developing medaka eggs. *Biol. Bull.* **183:** 70–77.

Fluck, R. A., A. L. Miller, V. C. Abraham, and L. F. Jaffe. 1994. Calcium buffer injections inhibit ooplasmic segregation in medaka eggs. *Biol. Bull.* **186:** 254–262.

Gard, D. L. 1991. Organization, nucleation, and acetylation of microtubules in *Xenopus laevis* oocytes: a study by confocal immunofluorescence microscopy. *Dev. Biol.* **143:** 346–362.

Gard, D. L. 1994. γ-tubulin is distributed asymmetrically in the cortex of *Xenopus* oocytes. *Dev. Biol.* **161:** 131–140.

Gilkey, J. C. 1983. Roles of calcium and pH in activation of eggs of the medaka fish, *Oryzias latipes. J. Cell Biol.* **97:** 669–678.

Grant, P., and J. F. Wacaster. 1972. The amphibian gray crescent region—a site of developmental information? *Dev. Biol.* **28:** 454–471.

Hamaguchi, M. S., and Y. Hiramoto. 1986. Analysis of the role of astral rays in pronuclear migration in sand dollar eggs by the Colcemid-UV method. *Dev. Growth & Differ.* **28:** 143–156.

Hiramoto, Y., M. S. Hamaguchi, Y. Nakano, and Y. Shoji. 1984. Colcemid uv-irradiation method for analyzing the role of microtubules in pronuclear migration and chromosome movement in sand-dollar eggs. *Zool. Sci.* **1:** 29–34.

Holwill, S., J. Heasman, C. R. Crawley, and C. C. Wylie. 1987. Axis and germ line deficiencies caused by u.v. irradiation of *Xenopus* oocytes cultured *in vitro. Development* **100:** 735–743.

Hunt, R. C., and A. A. Davis. 1990. Altered expression of keratin and vimentin in human retinal pigment epithelial cells *in vivo* and *in vitro. J. Cell. Physiol.* **145:** 187–199.

Ingold, A. L., S. A. Cohn, and J. M. Scholey. 1988. Inhibition of kinesin-driven microtubule motility by monoclonal antibodies to kinesin heavy chains. *J. Cell Biol.* **107:** 2657–2667.

Ivanenkov, V. V., A. A. Minin, V. N. Meshcheryakov, and L. E. Martynova. 1987. The effect of local microfilament disorganization on ooplasmic segregation in the loach *(Misgurnus fossilis)* egg. *Cell Differ.* **22:** 19–28.

Iwamatsu, T. 1973. On the mechanism of ooplasmic segregation upon fertilization in *Oryzias latipes. Jpn. J. Ichthyol.* **20:** 273–278.

Janson, L. W., and D. L. Taylor. 1993. *In vitro* models of tail contraction and cytoplasmic streaming in amoeboid cells. *J. Cell Biol.* **123:** 345–356.

Jeffery, W. R. 1984. Pattern formation by ooplasmic segregation in ascidian eggs. *Biol. Bull.* **166:** 277–298.

Katow, H. 1983. Obstruction of blastodisc formation by cytochalasin B in the zebrafish, *Brachydanio rerio. Dev. Growth & Differ.* **25:** 477–484.

Knapp, L. W., W. M. O'Guin, and R. H. Sawyer. 1983. Drug-induced alterations of cytokeratin organization in cultured epithelial cells. *Science* **219:** 501–503.

Ladrach, K. S., and J. R. LaFountain, Jr. 1986. Malorientation and abnormal segregation of chromosomes during recovery from Colcemid and Nocodazole. *Cell Motil. Cytoskel.* **6:** 419–427.

Pasdar, M., Z. Li, and K. A. Krzeminski. 1992. Desmosome assembly in MDCK epithelial cells does not require the presence of functional microtubules. *Cell Motil. Cytoskel.* **23:** 201–212.

Rafferty, N. S., S. Zigman, T. McDaniel, and D. L. Scholtz. 1993. Near-uv radiation disrupts filamentous actin in lens epithelial cells. *Cell Motil. Cytoskel.* **26:** 40–48.

Sakai, Y. T. 1964. Studies on the ooplasmic segregation in the egg of the fish *Oryzias latipes.* I. Ooplasmic segregation in egg fragments. *Embryologia* **8:** 129–134.

Sakai, Y. T. 1965. Studies on the ooplasmic segregation in the egg of the fish *Oryzias latipes.* III. Analysis of the movement of oil droplets during the process of ooplasmic segregation. *Biol. Bull.* **129:** 189–198.

Sawada, T., and K. Osanai. 1981. The cortical contraction related to the ooplasmic segregation in *Ciona intestinalis* eggs. *Wilhelm Roux's Arch. Dev. Biol.* **190:** 208–214.

Scharf, S. R., and J. C. Gerhart. 1980. Determination of the dorsal-ventral axis in eggs of *Xenopus laevis:* complete rescue of UV-impaired eggs by oblique orientation before first cleavage. *Dev. Biol.* **79:** 181–198.

Shimizu, T. 1982. Ooplasmic segregation in the *Tubifex* egg: mode of pole plasm accumulation and possible involvement of microfilaments. *Wilhelm Roux's Arch. Dev. Biol.* **191:** 246–256.

Sluder, G. 1976. Experimental manipulation of the amount of tubulin available for assembly into the spindle of dividing sea urchin eggs. *J. Cell Biol.* **70:** 75–85.

Webb, T. A., and R. A. Fluck. 1993. Microfilament- and microtubule-based movements during ooplasmic segregation in the medaka fish egg *(Oryzias latipes). Mol. Biol. Cell* **4:** 274a.

Yamamoto, T. 1967. Medaka. Pp. 101–111 in *Methods in Developmental Biology.* F. M. Wilt and N. K. Wessells, eds. Thomas Y. Crowell Company, New York.

Youn, B. W., and G. M. Malacinski. 1980. Action spectrum for ultraviolet irradiation inactivation of a cytoplasmic component(s) required for neural induction in the amphibian egg. *J. Exp. Zool.* **211:** 369–377.